

Posttranscriptional RNA Modifications: Playing Metabolic Games in a Cell's Chemical Legoland

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Nature combines existing biochemical building blocks, at times with subtlety of purpose. RNA modifications are a prime example of this, where standard RNA nucleosides are decorated with chemical groups and building blocks that we recall from our basic biochemistry lectures. The result: a wealth of chemical diversity whose full biological relevance has remained elusive despite being public knowledge for some time. Here, we highlight several modifications that, because of their chemical intricacy, rely on seemingly unrelated pathways to provide cofactors for their synthesis. Besides their immediate role in affecting RNA function, modifications may act as sensors and transducers of information that connect a cell's metabolic state to its translational output, carefully orchestrating a delicate balance between metabolic rate and protein synthesis at a system's level.

Introduction

Basic metabolic pathways use ubiquitous metabolites and coenzymes to transfer methyl groups, acetyl groups, amino acids, isoprenoids, sugars, phosphate, and the like. Many metabolite-RNA conjugates are known, or likely, to enhance the chemical functionality of their targets (Figure 1). Despite significant advances, technical bottlenecks have throttled progress in the modification field, including (1) the lack of sensitive analytic tools for the chemical or physicochemical identification and detection of modified nucleotides in limiting sample amount and (2) the absence of focused interest, by bioorganic chemists, to synthesize and generate authentic standards for the selective detection of existing as well as newly discovered modifications. However, recent developments in mass spectrometry and nucleoside chemistry have allowed more sensitive detection and quantification (Li and Limbach, 2012). Concomitantly, interest has resurfaced and connections are being made between tRNA modifications, a cell's overall stress response, cell development, and a cell's protein synthesis capacity (Chan et al., 2010, 2012).

Renewed interest also has been driven by the advent of methods for transcriptome-wide detection of simple RNA modifications, for example, 6-methyladenosine (m^6A) (Dominissini et al., 2012; Meyer et al., 2012), 5-methylcytosine (m^5C) (Squires et al., 2012), and inosine (Sakurai et al., 2010). Significant crosstalk from the DNA field also has promoted interest in RNA modifications, mainly arising from "newly discovered" DNA modifications that deservedly received enormous attention in the field of epigenetics (Calo and Wysocka, 2013).

In this review, we highlight modifications that depend on building blocks from several interconnected metabolic pathways and as such may help coordinate protein synthesis and metabolism. Possible relationships have been established between tRNA modifications and environmental changes. Here, we emphasize that although such connections have been made under conditions of environmental stress (i.e., oxidative stress), these con-

nections may not be limited to environmental extremes and could be part of a cell's normal program of growth.

Combinatorial Modifications and Modification Cascades

With increasingly complex modifications also comes sophisticated chemistry, presenting a synthetic challenge for the organic chemist. Nature, as usual, has elegantly solved chemical complications by breaking down the task into a series of simple steps, each catalyzed by specific enzymes. It is now clear that the overwhelming chemical diversity found in nucleoside modifications is actually created by versatile combinations of a limited subset of chemical reactions, e.g., group transfer reactions.

In most group transfer reactions, various nucleophilic sites in RNA are modified by activated variegated electrophiles that are part of the cell's typical metabolic repertoire and as such have coevolved with specific enzymes and catalytic motifs. The range of chemical reactions is not restricted to simple nucleophilic substitution but extends to oxidative reactions and C-H activation by transition metals, thiolation, selenation, amidation, and esterification as well as Michael, Schiff, and Mannich chemistries. This has led to modification diversity, where some modifications take place at several sites of a given standard nucleoside in a combinatorial manner, with little crosstalk between the progress at two given sites and with each pathway proceeding independently of the other. For example, two pathways operate rather independently at positions 2 and 5 of the uracil ring (Figure 2A). Thiolation at position 2 may precede or follow the synthesis of the side chain at position 5. As a result, these "orthogonal" transformations are interchangeable in their order of occurrence. Thiolation also may be followed by further modifications at the 2-position, e.g., geranylation. Methylation at the ribose 2'-OH position also occurs at several uridine derivatives, further compounding the variety of generated permutations. Among these, and regardless of the actual reaction mechanism, which we discuss below, C5-substituted uridines

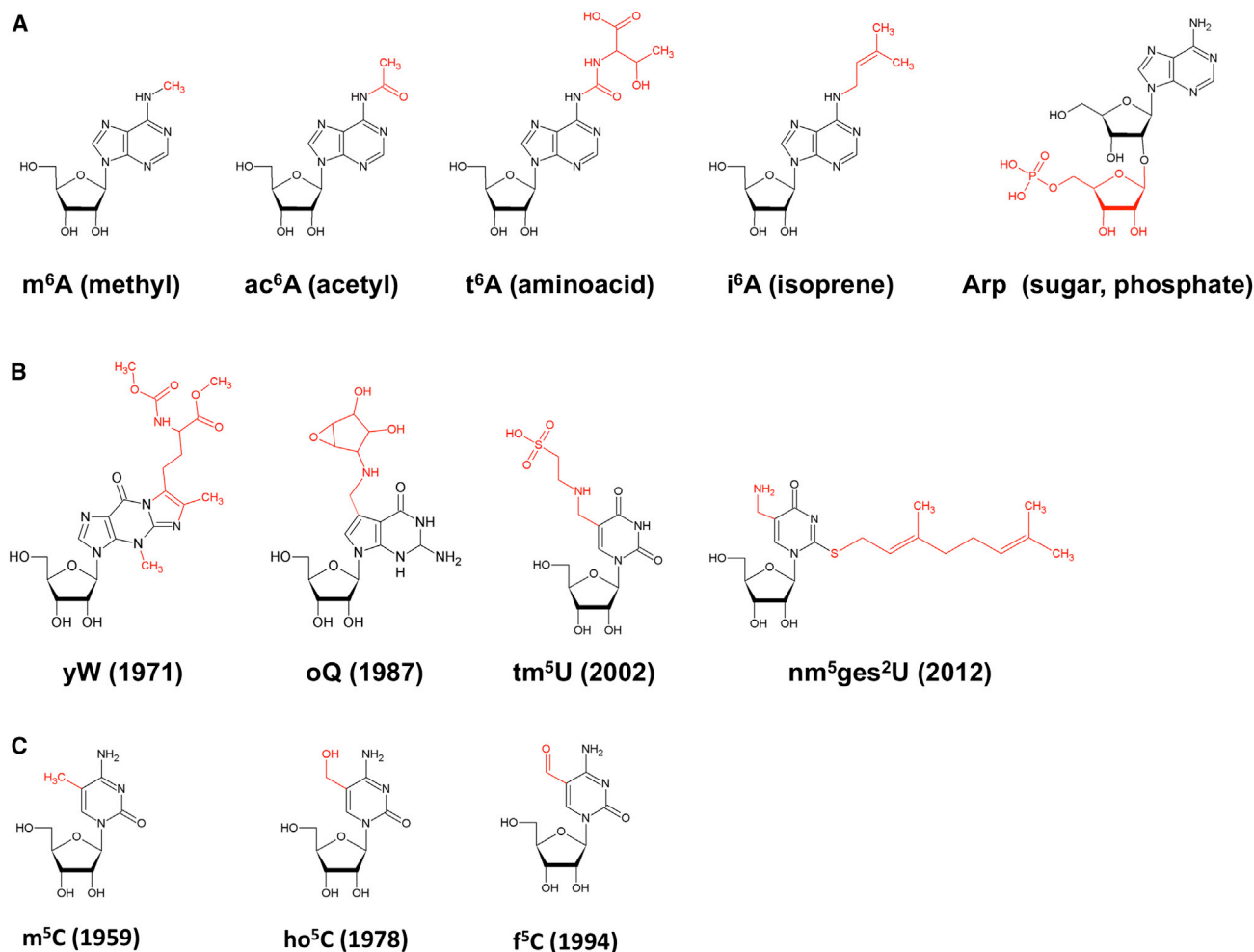


Figure 1. Selected Modified Ribonucleosides

(A) Adenosine modifications resulting from conjugation to ubiquitous electrophilic metabolites.

(B) Chemically sophisticated hypermodifications discovered throughout the past five decades. The year of publication is given in parentheses.

(C) The C5-modified cytidines related to so-called epigenetic DNA modifications recently discovered.

ac⁶A, 6-acetyladenosine; Arp, 2'-O-riboseyladenosine phosphate; oQ, epoxyqueuosine; tm⁵U, 5-taurinomethyluridine; nm⁵ges²U, 5-aminomethyl-2-geranyluridine; ho⁵C, 5-hydroxycytidine; f⁵C, 5-formyluridine.

are the largest contributors to the hitherto described chemical diversity (Machnicka et al., 2013).

In contrast to the partially “random” order of uridine modification (Figure 2A), other hypermodifications are executed in precise order, since the reaction steps, rather than being orthogonal, directly build on one another. For example, the biosynthesis of wybutosine (yW) (Figure 2B), in clear contrast to the wobble uridine pathways, splits up into several branches, but there is no reunification of any two branches, meaning that a substrate is committed to a defined sequence of modification steps after entering a branch. Thus, modifications can occur independently of each other between sites or be part of cascades at individual sites or even at different sites.

Fancy Chemistry at the Business End: The tRNA Anticodon

By far the greatest diversity of hypermodified nucleotides occurs at positions 34 and 37 of the anticodon of tRNAs. Modifications

at these positions presumably evolved both to enhance base-pairing flexibility during wobble decoding as needed (via position 34) and to ensure reading frame maintenance (by modifications at position 37) (El Yacoubi et al., 2012; Gustilo et al., 2008). The key roles played by anticodon modifications can be highlighted here by examples of enzymatic machineries that generate four well-studied anticodon modifications: (1) C5 methyluridine derivatives and queuosine (Q) at the wobble nucleotide (first position of the anticodon) and (2) yW derivatives and hypermodified adenosines neighboring the anticodon (position 37) (Figure 4).

Key Modifications at a tRNA's Corporate Headquarters: Methyluridine Derivatives and Q at Position 34

Together, the uridine at position 37 of the anticodon loop (U₃₄) modifications tend to increase the stability of the tRNA-mRNA interaction during decoding by increasing anticodon rigidity and by serving as determinants of amino acylation, translation

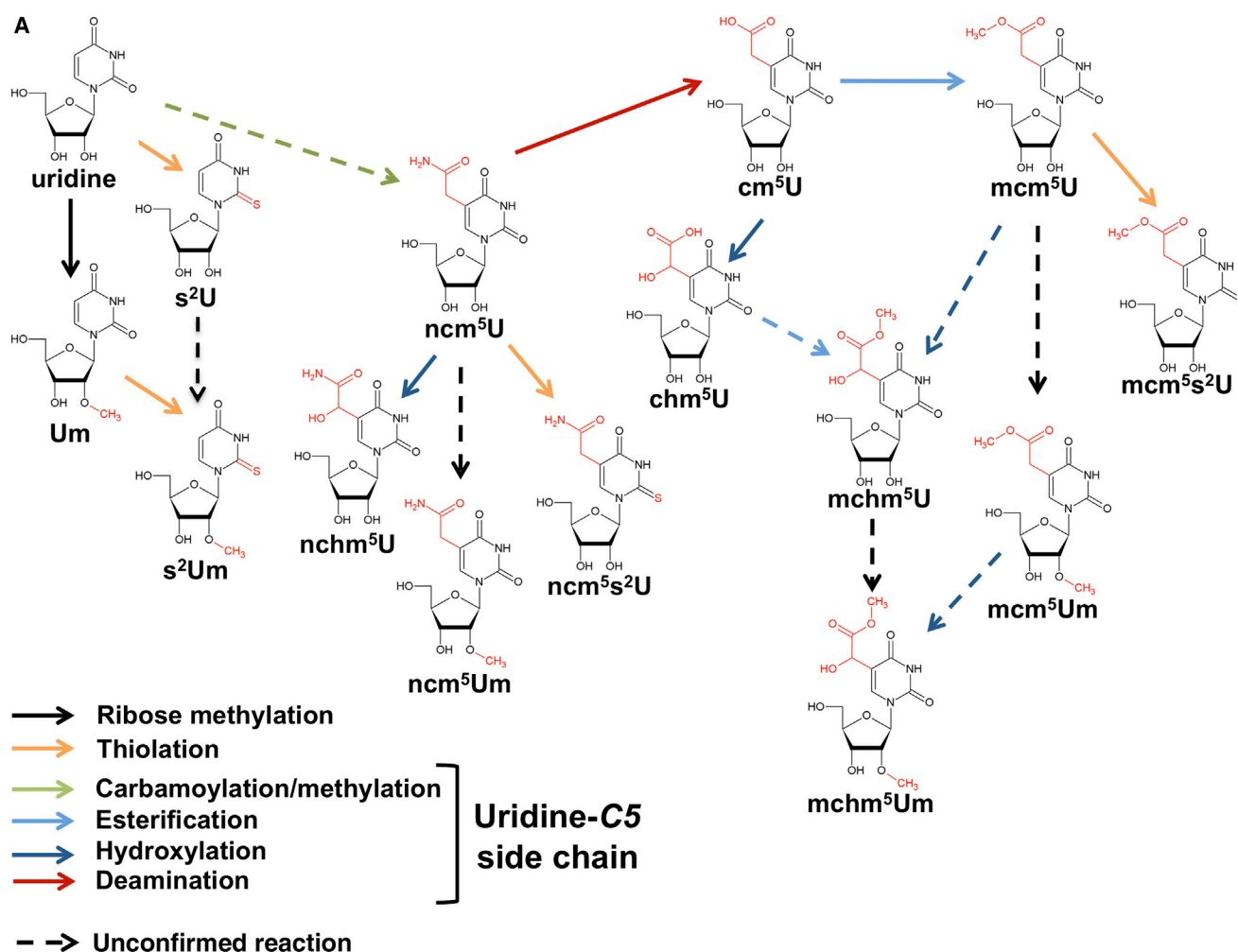


Figure 2. Hypermodification Pathways

(A) Eukaryotic modification network of uridines at the wobble position 34. The weave results from the overlay of independently operating modification pathways that modify at positions 2'-O of the ribose, C5 of the uridines, or O₂ of the uridines. The lack of a fixed order of action generates a multitude of permutations. (B) Biosynthesis pathway of yW derivatives. Note that transformations occur in a defined serial manner. Since branching pathways do not reunite, the pathway by which a given modification is generated is unambiguous. Numbers in parentheses refer to the TYW1–TYW5 enzymes involved in each step and referred to in the main text. Question marks (?) indicate reactions for which the given enzyme is not known.

Um, 2'-O-methyluridine; s²Um, 2'-O-methyl-2-thiouridine; ncm⁵U, 5-carbamoylmethyluridine; nchm⁵U, 5-carbamoylhydroxymethyluridine; ncm⁵Um, 5-carbamoylmethyl-2'-O-methyluridine; ncm⁵s²U, 5-carbamoylmethyl-2-thiouridine; chm⁵U, 5-carboxyhydroxymethyluridine; cm⁵U, 5-carboxymethyluridine; mcm⁵U, 5-methoxycarbonylmethyluridine; mchm⁵U, 5-(carboxyhydroxymethyl)uridine methyl ester; mchm⁵Um, 5-(carboxyhydroxymethyl)-2'-O-methyluridine methyl ester; mcm⁵Um, 5-methoxycarbonylmethyl-2'-O-methyluridine; mcm⁵s²U, 5-methoxycarbonylmethyl-2-thiouridine; m¹G, 1-methylguanosine; imG-14, 4-demethylwyosine; imG2, isowyosine; imG, wyosine; mimG, methylwyosine; OHyWx, undermodified hydroxywybutosine; o₂yW, peroxywybutosine; OHyWy, methylated undermodified hydroxywybutosine; yW-86, 7-aminocarboxypropyl demethylwyosine; yW-72, 7-aminocarboxypropylwyosine; yW-58, 7-aminocarboxypropylwyosine methylester.

efficiency, and fidelity (Gustilo et al., 2008). As mentioned, modifications of U₃₄ (first anticodon position), may start with thiolation of O₂, or with alkylation at C5 (Figure 2A). In the Bacteria, the end product is either 5-carboxymethylaminouridine (cmnm⁵U) or 5-methylaminomethyluridine (mnm⁵U). In mitochondria, owing to their bacterial ancestry, similar modifications occur, with mammals representing a notable exception where the final product involves the incorporation of the amino acid taurine to form 5-taurinomethyluridine (Suzuki et al., 2001) (Figure 1B). In eukaryotes, different versions of these modifications are then obtained by further 2'-O-methylation and/or 2-sulfurylation to X⁵-methyl-2-thio-2'-O-methyluridine uridine, where

X indicates any hypermodification of 5-methyluridine (m⁵U), for example, mnm⁵U (Figure 2A) (Armengod et al., 2012). Early studies in Bacteria implicated the involvement of a heterotetrameric enzyme complex formed by the proteins TRME (mnmE) and GidA in C5 modifications (Brégeon et al., 2001). Mutation of any of these genes leads to the complete disappearance of the C5 modifications. The crystal structure of TRME, a guanosine triphosphate (GTP)-binding protein, suggested that it could use 5-formyltetrahydrofolate, thus a C1 moiety at the oxidation state +II (5-formyltetrahydrofolate, corresponding to an equivalent of formic acid) as the source of the C1 moiety necessary for the first step of the reaction (Scrima et al., 2005). However,

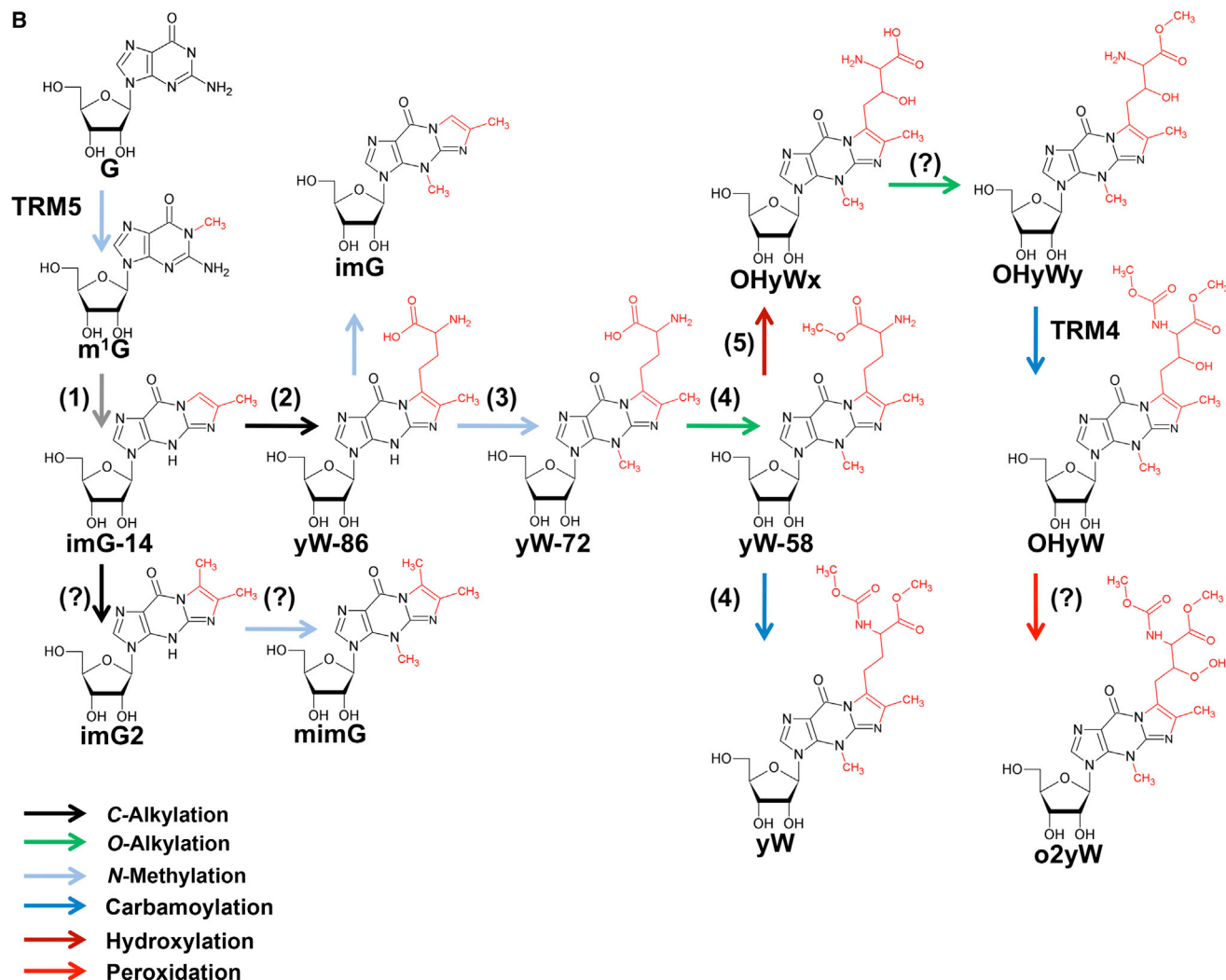


Figure 2. (continued).

several studies, including a biochemical reconstitution of the first step of the reaction, demonstrated a requirement for TRME/GidA and 5,10-methylene tetrahydrofolate (THF), thus a C1 donor at the oxidation state 0 (corresponding to an equivalent of formaldehyde) (Moukadiri et al., 2009) (Figure 5). This step is then followed by glycine addition by the second enzyme in the pathway, TRMC (MnmC1, MnmC2). GTP was proposed to effect a conformational change in the 5,10-methylene THF-binding site (Prado et al., 2013). This change is needed to bring together the C1 moiety of 5,10-methylene THF and the C5 position of uridine that are otherwise separated by a distance of 11 Å in the TRME crystal structure. Although the actual reaction mechanism has not been conclusively proven, structural and biochemical data as well as the analogy to related enzymes imply that TRME forms a covalent bond by Michael addition of a cysteine thiolate to C6 of U₃₄. This leads to activation of the C5 position and sets it on path for a nucleophilic attack onto the C1 electrophile (Meyer et al., 2009). The overall mechanism involves redox cofactors flavin adenine dinucleotide (FAD) and possibly nicotin-

amide adenine dinucleotide (oxidized form), but their precise oxidation state and role are yet to be determined. GidA also is involved in the glycine addition step to form cmnm⁵U (Figure 5). In other cases where mnm⁵U is found instead, TRMC removes the two-carbon skeleton of the glycine residue from cmnm⁵U in an FAD-dependent manner and further methylates the resulting 5-aminomethyluridine (nm⁵U) to mnm⁵U (Figure 3) (Armengod et al., 2012; Prado et al., 2013).

A different situation occurs in the eukaryotic cytoplasm, where C5 of U₃₄ is usually hypermodified to methoxycarbonylmethyluridine. Although lack of an in vitro system has hampered the biochemical elucidation of the reaction mechanism, ample genetic data link the enzymes involved in this pathway to the so-called elongator complex (Elp) (Huang et al., 2005; Mehlgarten et al., 2010). Elp is comprised of six proteins in yeast (Elp1–Elp6), originally identified as subcomplexes that serve as accessory factors to RNA polymerase II. Orthologs of Elp2–Elp4 exist in humans, with two additional proteins that are presumably analogous to Elp5 and Elp6 from yeast (Hawkes et al.,

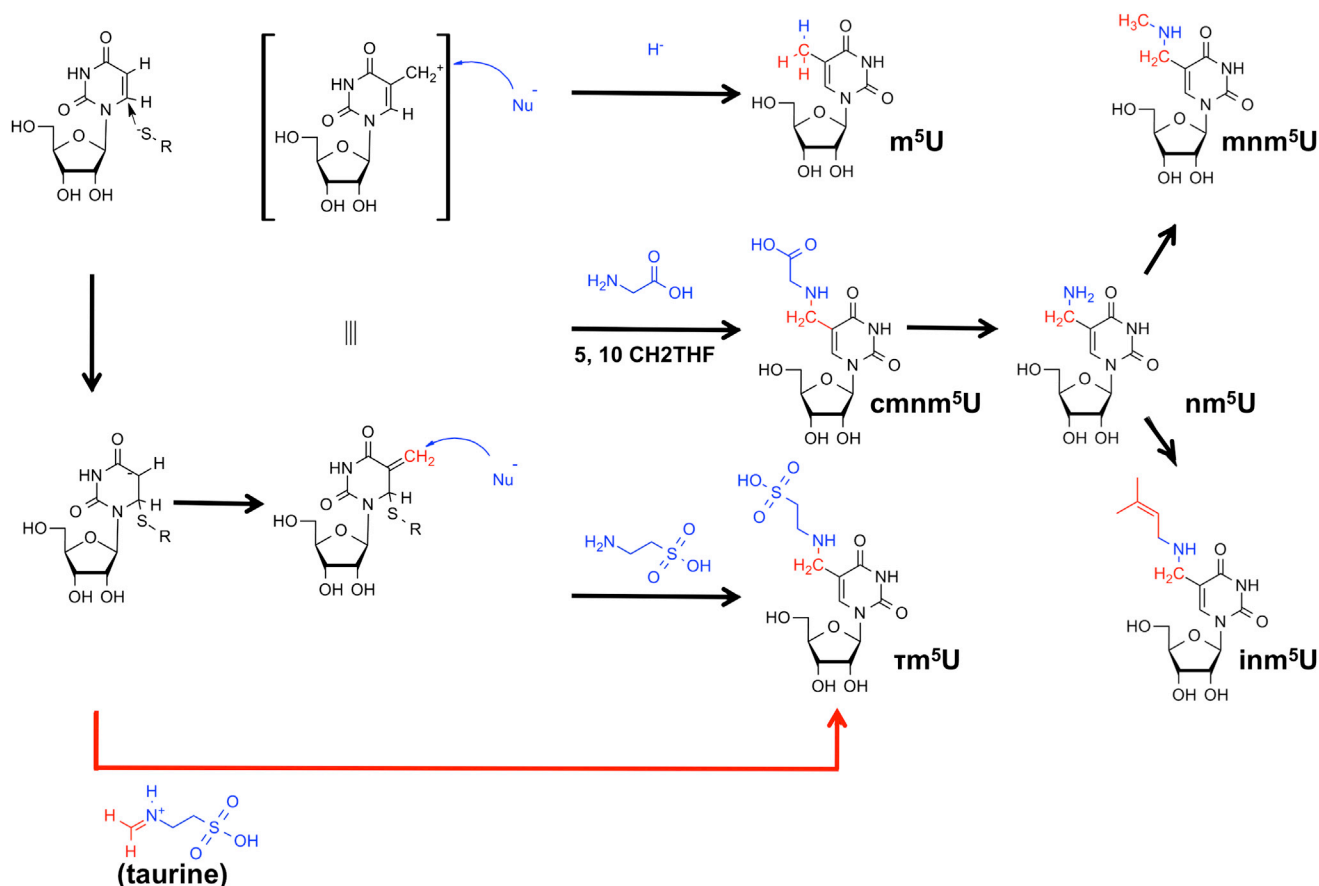


Figure 3. Chemical Strategies for C5 Modification of Uridines in Bacteria

The C5 position of uridines is rendered nucleophilic by Michael addition of a cysteine thiolate to the 5,6 double bond. 5,10-Methylene THF (5, 10 CH₂THF) provides a C7-body at the oxidation state of formaldehyde that acts as electrophile (red), leading to an electrophilic 5-methyleneuridine intermediate. This intermediate can be attacked by a variety of nucleophiles (blue) that may include hydride (from THF) or by different amino acids, such as Gly (Bacteria) or taurine (metazoan mitochondria). After dealkylation of cmnm⁵U, the resulting nm⁵U can be methylated to mnm⁵U or prenylated to inm⁵U. The latter reaction is unconfirmed.

tm⁵U, 5-taurinomethyluridine; mnm⁵U, 5-methylaminomethyluridine; inm⁵U, 5-isopentenylaminomethyluridine.

2002). In yeast, mutations in any of the Elp genes lead to complete ablation of 5-methoxycarbonylmethyluridine (and 5-carbamoylmethyluridine) formation in tRNA (Huang et al., 2005). Together, these observations are most remarkable considering that the same complex plays important roles in histone acetylation and transcription regulation (Svejstrup, 2007).

Despite apparent variance in mechanisms between Bacteria and Eukarya, the eight chemically distinct modifications occurring at C5 of wobble uridines (xm⁵U₃₄, where x indicates any hypermodification of m⁵U, for example, mnm⁵U; Figure 2A) all share a common 5-carboxymethyl structure at their core. As a reflection of the variegated chemistry involved, the enzymes involved require substrates such as S-adenosylmethionine (SAM), glycine, taurine, and folate that are derived from different metabolic pathways. The modification pathway is thus part of a network of metabolic interactions, and beyond this, ties into components, such as Elp, that because of their function in transcription, play critical roles in affecting gene expression.

Bacterial tRNAs also undergo further modifications at U₃₄, including the thiolation reaction that replaces the exocyclic oxygen at C2 in tRNA^{Lys}_{UUU}, tRNA^{Gln}_{UUG}, and tRNA^{Glu}_{UUC}. Despite

affecting the same nucleotide, 2-thiouridine (s²U) and xm⁵U (Figure 2A) formation are independent of each other. In terms of evolution, the enzymes responsible for s²U formation evolved independently in the eukaryotic and bacterial lineages. Both sets of enzymes exist in eukaryotic cells where the cytoplasmic pathway is uniquely eukaryotic, yet the mitochondrial pathway is still bacterial in nature. Cytoplasmic thiolation to 2-thiouridine at position 34 of the anticodon requires an enzymatic cascade performed by a series of ubiquitin ligase-like proteins (Leidel et al., 2009). In mitochondria, the complete pathway has not been elucidated but is known to include the MTU1 (TRMU) enzyme, a homolog of the mnmA enzyme from Bacteria (Umeda et al., 2005). The bacterial system also includes a series of small proteins that catalyze a modification cascade starting with transfer of the sulfur from cysteine and involvement of IscS, the same desulfurase enzyme essential for catalyzing sulfur incorporation during Fe-S cluster assembly into many proteins, including many modification and metabolic enzymes (as discussed below) (Shigi et al., 2008).

When tRNAs are encoded with a guanosine at the wobble position (G₃₄), in the context of a GUN anticodon, where N

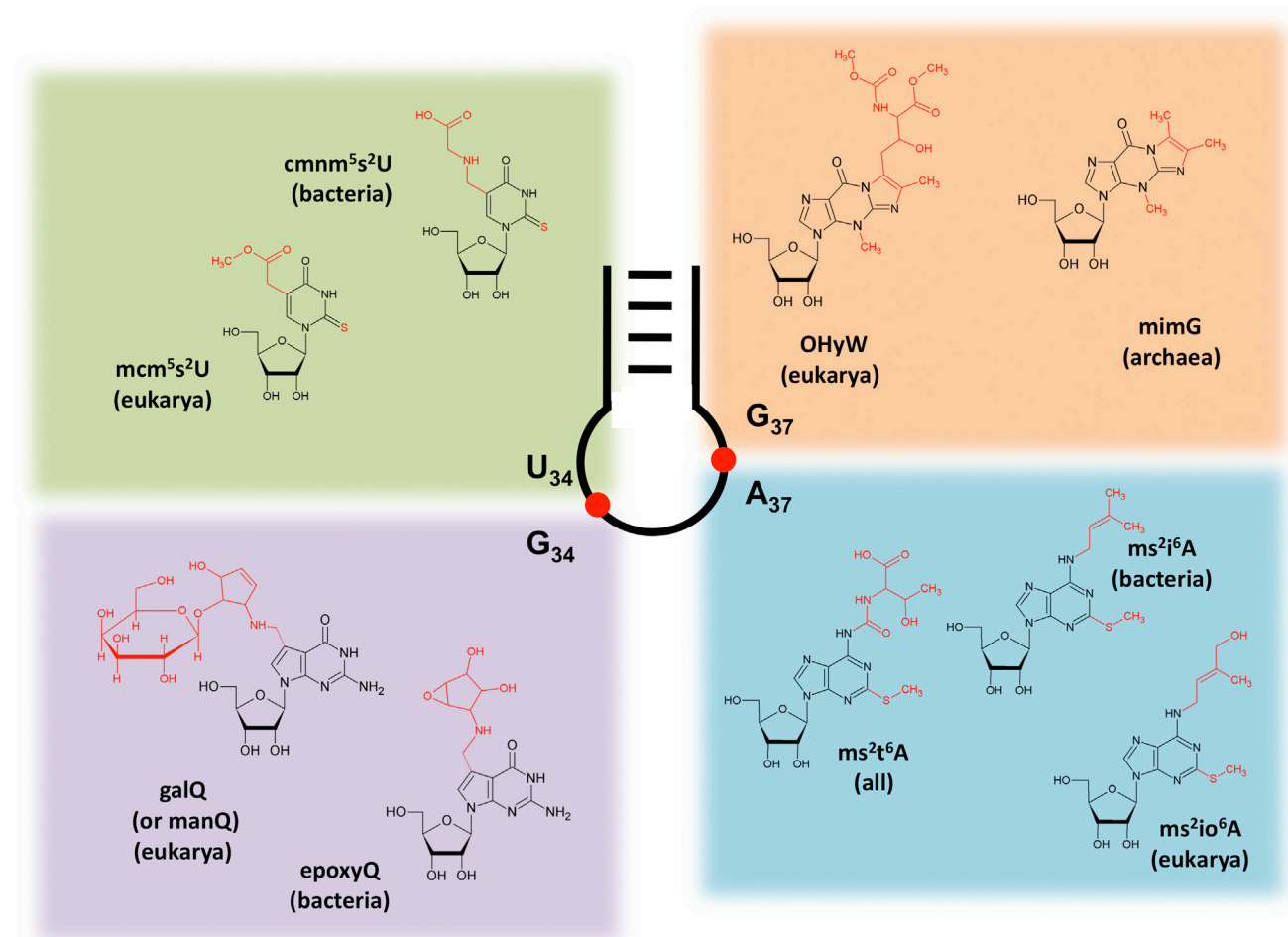


Figure 4. Hypermodifications at Positions 34 and 37 in the Anticodon Loop

Positions 34 and 37 of the anticodon loop undergo by far the largest diversity of posttranscriptional modifications. Highlighted are modified uridines (upper left panel) and guanosines (lower left panel), ubiquitous hypermodifications ensuring correct decoding at the wobble position. Sophisticated purine modifications found at position 37 (upper and lower right panels) play roles in reading frame maintenance.

cmnm⁵s²U, 5-carxymethylaminomethyl-2-thiouridine; galQ, galactosylqueuosine; epoxyQ, epoxyqueuosine; mimG, methylwyosine; ms²t⁶A, N⁶-2-methylthio-N⁶-threonylcarbamoyladenine; ms²io⁶A, 2-methylthio-N⁶-(cis-hydroxyisopentenyl) adenosine.

represents any nucleotide, this nucleotide is usually hypermodified to Q (yeast representing one of the few exceptions). This includes tRNA^{Asn}, tRNA^{Asp}, tRNA^{His}, and tRNA^{Tyr}, but tRNA^{Asp} and tRNA^{Tyr} are further glycosylated to mannosyl Q-tRNA (manQ₃₄) and galactosyl Q-tRNA (galQ₃₄), respectively (Chen et al., 2011) (Figure 4). Regardless of the tRNA species, in all cases Q plays critical roles in translation. Interestingly, the route to Q formation in tRNA differs markedly in Bacteria and Eukarya. In Bacteria, five enzymatic steps are necessary for the de novo biosynthesis of 7-aminomethyl-7-deazaguanine (preQ; Figure 4) that is then used to replace guanosine in the tRNA substrates by the enzyme tRNA-guanine transglycosylase (TGT) (Chen et al., 2011). Once on the tRNA, preQ is further modified to Q and eventually glycosylated, as described above, in tRNA^{Asp} and tRNA^{Tyr} but not tRNA^{Asn} or tRNA^{His}. All eukaryotes, with the exception of yeast, where TGT is missing, contain Q in tRNA; however, these organisms do not synthesize preQ and can only incorporate queuine, the free base form of Q, through salvage from nutrients

in the media, where queuine is preformed by the bacterial gut microflora (Iwata-Reuyl, 2003). These differences in substrate specificity led to the proposal that both pathways were the result of convergent evolution. However, recent crystallographic and biochemical evidence strongly supports the view that in fact they are evolutionarily divergent. What is not clear is whether eukaryotes once had the preQ pathway and lost it, or alternatively, never had it. It stands to reason that the former explanation is the likely explanation and the result of the intimate interaction between Bacteria and Eukarya in their crossing evolutionary paths (Chen et al., 2011).

In terms of cellular physiology, in Bacteria, lack of Q leads to issues of translational efficiency, best manifested in a reduction in virulence in some pathogens. This pathway has thus been proposed as an antibacterial target (Goodenough-Lashua and Garcia, 2003). In Eukarya, beyond translation, laboratory animals deficient in TGT grow normally, but if the nonessential amino acid tyrosine is left out of their feed, they die within 18 days

(Rakovich et al., 2011). The early symptoms of tyrosine-less/Q-less diet easily disappear with the addition of tyrosine to growth media. The defect has been ascribed to an inability of Q-less mice to synthesize tyrosine from phenylalanine by phenylalanine hydrolase (PAH), presumably due to low levels of tetrahydrobiopterin (BH4), an essential cofactor for PAH. However, it is still not clear why this imbalance in BH4 levels only occurs in the absence of Q (Rakovich et al., 2011).

Also different between Bacteria and Eukarya is the nature of the TGT enzyme itself. The bacterial enzyme is either monomeric or homomultimeric, depending on the study (Chen et al., 2011). The eukaryal enzyme, at least in mice, requires two nonidentical subunits derived from alternative splicing of the TGT gene (Boland et al., 2009). Neither subunit by itself can catalyze the reaction, yet mixing recombinant versions of the two robustly reconstitutes activity in vitro. Presumably, one of the subunits is used in salvaging queuine from Q monophosphate resulting from tRNA turnover, whereas the second subunit is in charge of catalyzing the Q exchange on the tRNA. Just as complex is the localization of the two eukaryotic TGT subunits. Queuosine is found in both cytoplasmic and mitochondrial tRNAs in mice, yet both subunits appear to localize to mitochondria (Boland et al., 2009). The how or why of this unusual localization remains an open question.

The Importance of Good Neighbors: Hypermodifications of Position 37

The only other position in the anticodon of tRNAs to undergo hypermodification is position 37, neighboring the anticodon sequence (Figure 4). This position is always an encoded purine that in all three domains of life is almost always modified. The evolutionary conservation of this modified position signals its importance in stabilizing anticodon-codon interaction during decoding. It also plays critical roles in reading frame maintenance. In general, modifications at position 37 help maintain and open loop conformation, preventing base pairing with neighboring nucleotides on the other side of the anticodon loop (uridine 33) and helping formation of a canonical anticodon loop structure important for decoding (Cabello-Villegas et al., 2002).

In most tRNAs an encoded G₃₇ is methylated at the base to form 1-methylguanosine (m¹G), which by itself plays critical roles in aminoacylation and/or translational accuracy. Because m¹G is found in tRNAs in all domains of life, it was probably present in the last common ancestor, already serving important functions in establishment of the genetic code early in evolution (Björk et al., 2001). However, 1-methylguanosine at position 37 of the anticodon loop (m¹G₃₇) also serves as the chemical platform for additional group additions in the case of tRNA^{Phe} of Archaea and Eukarya. In these organisms, m¹G is the first step in the formation of yW in Eukarya and wyosine and derivatives in Archaea (de Crécy-Lagard et al., 2010; Noma and Suzuki, 2006). Synthesis of this nucleoside(s) requires a series of enzymatic steps starting with formation of a tricyclic ring on the methylated guanosine (Figure 3A). Interestingly, the eukaryotic TYW1 protein contains a flavin mononucleotide (FMN)-binding domain that is presumably important for the reduction of the essential [4Fe-4S] cluster proposed to be critical for catalysis. Although this reaction has not been reconstituted in vitro in the eukaryotic system, it was shown that deletion of the TYW1 gene leads to

disappearance of yW with a concomitant appearance of m¹G in tRNA^{Phe} (Björk et al., 2001).

In Eukarya, after the TYW1-catalyzed reaction, TYW2 transfers the α -amino- α -carboxypropyl (acp) group from SAM, forming the intermediate yW-86, so called because it differs from fully formed yW by 86 daltons (Noma et al., 2006) (Figure 2B). This intermediate is then methylated at the original purine ring N-3 position by TYW3 to form yW-72; the acp group is then methylated by TYW4 to yW-58 and subsequently methoxycarbonylated by the same enzyme to form yW (Figure 2B). This last step is particularly interesting in that it involves CO₂ fixation, leading to the suggestion of a new role for SAM (Suzuki et al., 2009). However, all energetic details for this reaction have not been fully clarified.

An additional question is the origin of the different methylation and methoxycarbonylation activities that involve the synthesis of yW. Curiously, at least in the case of TYW4, the crystal structure offers important clues. This enzyme contains a domain in common with protein phosphatase methyltransferase 1 (PPM1) that methylates protein phosphatase 2A (PP2A) in a SAM-dependent manner. In TYW4, a nine amino-acid loop is present that has been replaced in PPM1 (Suzuki et al., 2009). This loop contains key positively charged residues important for substrate binding and is proposed to have change specificity from a protein (PP2A) to a tRNA^{Phe} substrate through evolution (Suzuki et al., 2009).

In Eukarya after the TYW1-catalyzed reaction, the C7 side chain differs in the extent of modification. Unlike yeast, in mammals the final product is the hydroxylated yW derivative hydroxywybutosine (OHyW) (Urbonavicius et al., 2009) (Figure 2B). Although this difference was known for many years, it was only recently reported that a fifth enzyme in the pathway exists, appropriately named TYW5. This enzyme belongs to the Jumoni C domain-containing family, typical of histone demethylases (Kato et al., 2011). Recent biochemical data have shown that TYW5 catalyzes an Fe(II)/2-oxoglutarate hydroxylation reaction in the final step of yW biosynthesis in humans. In addition, analogous to the fusion of TYW2, TYW3, and TYW4 in plants, in humans TYW4 and TYW5 also are fused, suggesting coupling of the last two reactions (Kato et al., 2011).

The variability in the end product of wyosine and derivatives is even more complex in Archaea. Although the reaction usually ends after the TYW3 step, due to the lack of genes encoding TYW4 and TYW5, various combinations of wyosine derivatives exist depending on the organism. For example, most Euryarchaeota lack TYW3, yet others have the acp side-chain replaced by a methyl group (isowyosine). In these Archaea the observation of a gene duplication of the TRM5 methylase (responsible for m¹G₃₇) has led to the suggestion that the duplicated gene product is responsible for this additional methylation, but this has to be formally proven (de Crécy-Lagard et al., 2010).

Perhaps the biggest puzzle in wyosine synthesis in Archaea is the absence of the highly conserved FMN-binding domain found in eukaryotic TYW1 (de Crécy-Lagard et al., 2010). This raises the question of what provides the function needed to reduce the essential Fe-S clusters. It has been suggested that in Archaea an additional protein, perhaps thioredoxin reductase, serves this function in *trans*. In addition the nature of the two-carbon compound needed to form the third ring had precluded

reconstitution of the TYW1 reaction until recently, when the use of dithionite as the source of reducing power in vitro established that pyruvate is the key substrate needed for ring closure (Young and Bandarian, 2011). Additional studies then showed that TYW1 from Archaea contains not one, but two, Fe-S clusters, whereby cluster I is proposed to bind SAM and mediate formation of a SAM radical that abstracts a proton from the methyl group of m¹G, creating the substrate radical that attacks pyruvate, forms the tricyclic wyosine, and releases CO₂ (Perche-Letuvée et al., 2012). Although pyruvate has not been validated as the two-carbon donor in eukaryotes, a similar mechanism is expected.

The presence of wyosine and derivatives is suggested to lead to a single functional outcome, hypermodification, which may provide base-stacking interactions that play a key function in reading frame maintenance (Urbanavicius et al., 2003). Why then are wyosine and derivatives only found at position 37 of tRNA^{Phe}? It may well be due to the nature of the phenylalanine codons, UUU and UUC, and their propensity for ribosome “slippage” (Atkins and Björk, 2009). These observations then lead to the possibility that the extent of tRNA^{Phe} modification at position 37 may correlate with the frequency of polyuridine slippery sequences in genomes. Why then use the yW strategy when m¹G alone can prevent unwanted frameshifting at most codons? The answer may rest in the idea of “frameshifting potential” (Waas et al., 2007), whereby, like in many viruses, frameshifting is used in a programmed manner perhaps to increase coding diversity. This then explains the variability of tRNA^{Phe} modification at G₃₇ ranging from no yW in *Drosophila* to hypermodification in mammals. Frameshifting potential implies that cells as part of their normal translation programs may exploit frameshifting as a regulatory mechanism and not just avoid it, at all cost, as a source of translational infidelity.

The only other anticodon loop hypermodifications occur when position 37 is an encoded adenosine. In all domains of life, this position is usually isopentenylated to form isopentenyl adenosine (i⁶A), and in Bacteria i⁶A can be further hypermodified to 2-methylthio-*N*⁶-isopentenyladenosine (ms²i⁶A) or 2-methylthio-*N*⁶-(*cis*-hydroxyisopentenyl) adenosine (ms²io⁶A) depending on the tRNA and the organism. The i⁶A system of Bacteria targets tRNAs for all codons with a “U” at the first position, whereas in Eukarya the tRNA substrates vary but are generally restricted to a smaller set of tRNAs. In terms of substrate specificity, the bacterial enzymes recognize A₃₆A₃₇A₃₈ as a major determinant for i⁶A formation, whereas in Eukarya what determines a good substrate depends once again on the organism, revealing a remarkable plasticity in substrate recognition by this family of enzymes (Lamichhane et al., 2011).

Whether the end product is i⁶A or the methylthiolated counterpart, the isopentenyl addition reaction is catalyzed by the product of the miaA gene in Bacteria (MOD5 in Eukarya); the enzyme catalyzes the transfer of the dimethylallyl moiety from dimethylallyl pyrophosphate to adenosine at position 37 of the anticodon loop (A₃₇) in various tRNAs, using Mg as the only required cofactor in vitro. In Bacteria, i⁶A can be further modified to ms²i⁶A or ms²io⁶A by a bifunctional enzyme encoded by miaB. Coincidentally, this enzyme also contains two Fe-S clusters analogous to the TYW1 enzyme of Archaea. Despite i⁶A and derivatives constituting bulky modifications, their function differs

from that of the previously described yW/wyosine derivatives in that rather than playing roles in frame maintenance, A₃₇ modifications are key determinants for codon-specific translation and therefore translation efficiency (Lamichhane et al., 2011).

For tRNAs decoding the ANN codons, it has been established for a long time that A₃₇ is universally modified to form *N*⁶-threonylcarbamoyladenosine (t⁶A) and can be further methylated at the base (e.g., *N*⁶-methyl-*N*⁶-threonylcarbamoyladenosine); this modification is also crucial to translational accuracy. In all systems, the t⁶A biosynthetic pathway requires Thr, ATP, and carbonate as substrates; in Bacteria it involves the Sua5/YrdC family of proteins and in Eukarya the Kae1/YgjD/Qri7 family (El Yacoubi et al., 2009). Despite the fact that each system has recruited different evolutionarily unrelated proteins to catalyze the reaction, minimally Sua5 and Qri (a Kae1 homolog) are required for t⁶A formation as shown in the mitochondrial system. Significantly, mutants in these families of proteins lead to pleiotropic effects in cells, including defects in transcription and genome stability (El Yacoubi et al., 2009).

The t⁶A story, besides its biological significance, also offers a cautionary tale when studying modifications. Despite its existence being accepted for more than 20 years, work showed that in fact in Bacteria (and perhaps in all organisms), the native modification is cyclic t⁶A (ct⁶A), a dehydrated form of t⁶A. It turns out that ct⁶A is so reactive that at neutral pH the cyclic side chain is destroyed. In line with this observation, an E1-like enzyme with ATPase activity, TcdA, has been identified as the dehydratase responsible for cyclization. Notably, ct⁶A is so reactive that it also can readily form adducts with the amines in Tris or ethanolamine buffers. Biologically, the lack of TcdA leads to respiratory defects in yeast, suggesting its particular importance in mitochondrial decoding (Miyachi et al., 2013).

Environmental Sensors and Metabolic Integrators

Efforts in the past few years have focused on the identification and characterization of the enzymes responsible for various modifications. New technologies have considerably accelerated the process, and in certain organisms, such as *Saccharomyces cerevisiae* and *Escherichia coli*, almost all of the tRNA modifications and corresponding enzymes have been cataloged. Many of the enzymatic reactions also have been established in vitro, and much progress has been made toward understanding different modification enzyme mechanisms. Even in cases where reactions have not been recapitulated in vitro, strong genetic evidence has linked several distinct proteins to a given modification. This knowledge also has benefited from progress in the structure determination of modification enzymes, providing further insight into their specificity and modes of substrate recognition. The next major challenge in the modification field is to establish how environmental changes affect modification sets and how these in turn get transduced into various cellular signals. The idea of a connection between tRNA modifications and environmental states dates back to several reports in the 1970s in which changes in tRNA modification content were correlated to how cells respond to various environmental stimuli, including exposure to UV light, challenge by phage infections, changes in growth rate, and changes in growth temperature (Emilsson et al., 1992). Back then, the technical challenges in

quantitatively assigning modifications to specific nucleotides and specific positions in a tRNA sequence forced these studies to remain mostly descriptive and could not push the ideas of modifications as environmental sensors much further.

Several subsequent reports have measured the impact of modification in response to temperature changes. A key observation here was the induction of a set of tRNA modifications at elevated temperatures in thermophiles (Kowalak et al., 1994). These studies followed the line of reasoning that given the important roles played by modifications in maintaining the stability of the folded L-shape tRNA, these may play particularly important roles in organisms that grow at high temperatures. Not surprisingly, the lack of some modifications, for example, 2-thiothymidine, led to temperature sensitivity in several bacteria. Likewise, the lack of pseudouridine 55 leads to a decrease in the levels of 2'-O-methylguanosine at position 18 of tRNA, and lack of both modifications leads to temperature sensitivity in *Thermus thermophilus* (Ishida et al., 2011). Because these modifications are important for necessary tertiary contacts between the D-loop and T Ψ C loops and for maintaining the tertiary fold of many tRNAs, their role in coping with elevated temperatures makes sense and therefore represents a modification-mediated adaptation to an extreme environment.

The question still remains how, more globally, modifications play roles in dealing with environmental extremes. To this end, while applying different stressors to yeast cells in the laboratory, liquid chromatography-tandem mass spectrometry approaches revealed that the global modification set of tRNAs changed in a dynamic way and depended on the specific stress (Chan et al., 2010). For example, the levels of m⁵C, N²-N²-dimethylguanosine, 2'-O-methylcytosine, and t⁶A changed in response to H₂O₂-induced oxidative stress. If an alkylating agent, such as methylmethanesulfonate (MMS), was used instead, a different set of modifications changed (including 1-methyladenosine, 3-methylcytosine, and 7-methylguanosine). Although these studies could not tell whether these changes were specific to tRNA sets, affected all tRNAs, or affected a single tRNA to different levels at a specific location, some of the modifications, such as t⁶A and m³C, are significant in that these affect subsets of tRNAs and only occur in the anticodon loop, perhaps more directly implicating modification dynamics to translational changes. Such effects on translation have indeed been reported by studies showing that tampering with TRM9 has significant effects on how cells cope with ionizing radiation or MMS (Chan et al., 2012). Since TRM9 is key in the formation of xm⁵U (Figure 2A), the observed phenotypes directly point to changes in translational efficiency of mRNAs rich in codons for which the modification is needed for decoding.

Along the same lines it was shown that, more specifically, increases in m⁵C methylation in tRNA^{Leu}_{CAA} were crucial to the cells response to oxidative stress. Remarkably, an increase in m⁵C levels induced by oxidative stress is coupled to translation of a TTG-rich mRNA encoding the ribosomal protein RPL22A, but not of an mRNA for a paralogous protein that has a lower use of the TTG codon (Chan et al., 2012). This observation is significant in that the ribosomal protein RPL22A has an established role in oxidative stress response. This observation speaks vol-

umes for the dynamic nature of modifications in response to environmental stress and in at least one respect, suggested by the authors, it implies a codon-specific remodeling of translational efficiency imparted by modifications.

Current work on the connection between modifications and environmental signals has concentrated on areas with a vast body of knowledge on how cells respond to certain well-known stresses, for example oxidative stress. However, response to stress caused by environmental extremes may just be one side of cellular modification dynamics. A careful look at the few hypermodification pathways discussed in this review quickly points to the systemic nature of modifications (Figure 5). Many modifications require cofactors and substrates that belong to seemingly disparate yet intricately interconnected metabolic pathways and as such, far and beyond stress, modification dynamics also may play a role in the adjustment and coupling of metabolic rates to tRNA function and protein synthesis as a means of overall cell homeostasis and during normal growth conditions. Clearly, many modifications borrow metabolites from other biosynthetic pathways in cells, and we view these as an additional level of metabolic integration. For example, formation of t⁶A (and derivatives) uses Thr that itself is made from Asx in Bacteria and salvaged from the media in humans (as an essential amino acid). In fact, in humans a potential connection between Thr and SAM has been reported whereby Thr and SAM metabolism are clearly coupled in determining the pluripotency of stem cells (Shyh-Chang et al., 2013). Thr-derived glycine, but not Ser-derived, contributes to 5-m-tetrahydrofolate levels and SAM generation. When Thr is limiting, there is an observed decrease in acetyl-coenzyme A (CoA) pools that, in turn, leads to a decrease in nicotinamide adenine dinucleotide. Thus, changes in Thr levels can affect production of reducing equivalents (Shyh-Chang et al., 2013). All these factors combined decrease embryonic stem cell growth and increase differentiation. Although these represent the result of cellular nutrient stress, clearly changing levels of SAM, Thr, and Gly indirectly affect several of the key hypermodifications discussed in the previous sections of this review. As such, nutrient response should involve the integration of metabolic signals and protein synthesis via tRNA modifications during normal cell growth, far and beyond the realm of nutritional stress. The same argument can be made for the biosynthesis of i⁶A that again requires the substrate dimethylallyl pyrophosphate (DMAPP). The DMAPP itself is derived from acetyl-CoA (Figure 5) that, in turn, may come from glycolysis via pyruvate or fatty acid beta oxidation. Again, this begs the question of how small physiological changes in acetyl-CoA levels may affect i⁶A biosynthesis and thus lead to reduction in the translation of certain mRNAs rich in codons that require i⁶A-containing tRNAs. Along these lines, a genetic screen in *S. cerevisiae* involving the MOD5 enzyme (responsible for i⁶A biosynthesis) revealed a competition between sterol and i⁶A biosynthesis, two pathways that require DMAPP, a substrate that is in limited supply in yeast (Benko et al., 2000). Similar arguments have been made for the possible connection between thiolation and Fe-S cluster assembly pathways in Bacteria and Eukarya that may extend to metabolic connections beyond tRNA maturation and may again involve seemingly unrelated pathways.

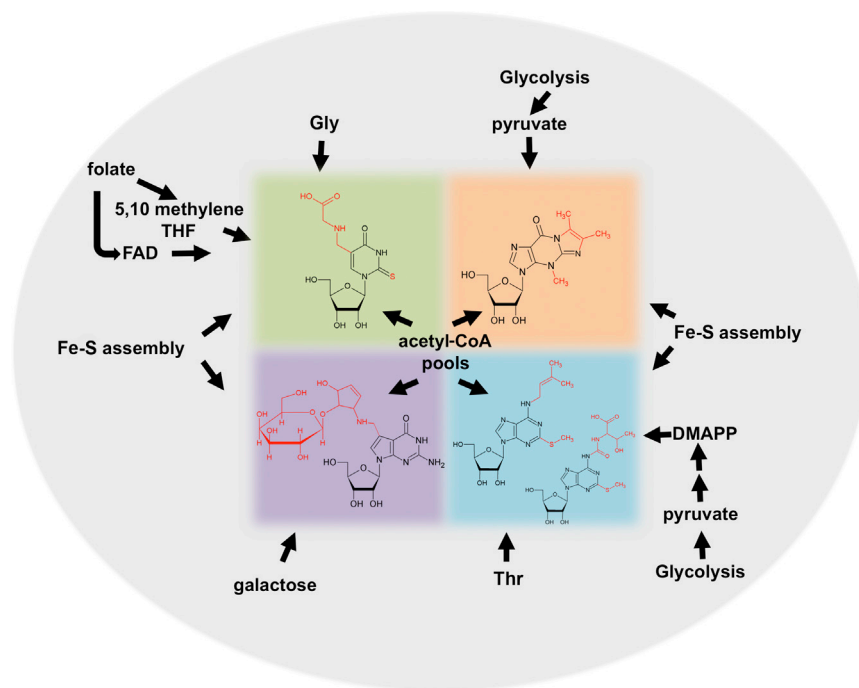


Figure 5. Various Metabolic Pathways Contribute Cofactors for Modification

The figure shows hypermodifications highlighted in this review that require building blocks from common metabolic pathways, for example, pyruvate derived from glycolysis. These interconnections may suggest coupling between metabolic and translational rates. The individual color boxes correspond to those modifications shown in Figure 4.

the hydrophobicity of yW led earlier to the isolation of large amounts of tRNA^{Phe} necessary for crystallography, eventually leading to the first X-ray structure of a tRNA (Kim et al., 1974; Robertus et al., 1974; Hoskinson and Khorana, 1965; Rajbhandary et al., 1967). With this in mind, future search strategies for new modifications may be tailored toward efficient affinity purification methods. For these there is ample inspiration in the literature, for example, the retention of S-containing modifications in Hg gels (Igloi, 1988) and vicinal diols by boronic acids (Igloi and Kössel, 1985).

Conclusions

Exploring the Limits of RNA Natural Product Synthesis: Modifications yet to Be Found

The preceding pages have highlighted the integrative nature of RNA modifications, in further support of the idea that these do not act alone but rather operate in a tangled web of interconnections that, in the context of the four canonical nucleotides, determines both their chemical diversity and modification enzyme specificity. This level of chemical integration has led us to suggest that the occurrence of subtle phenotypes, when approaching modification function genetically, has unfairly led to the conclusion that the impact of modification on cell function is minor, if any. However, it is possible that to a great degree lack of a single modification may be partially or fully compensated by the appearance of additional modifications in the tRNA that, in turn, may satisfy the function provided by the normally occurring but now missing modification. Thus, tackling to what extent modifications affect RNA function at a cellular scale is indeed a difficult business. Reductionism dictates system minimalization and the study of modifications as single entities, yet their effects cannot be fully realized without thinking about modifications in the context of the ever-changing cellular environment.

The identification of geranylated uridines (Dumelin et al., 2012) will certainly not be the last in a long line of discoveries that started in the 1950s and that produced surprises with remarkable steadiness, as illustrated by the discovery dates given in Figure 1. What may mark a new trend is the approach taken by the Liu laboratory: the search for new modifications by anticipation of what could be out there, while taking into consideration where and how one had to look for it, in chemical terms, rather than in what organism. Because geranylation makes RNA more lipophilic, geranylated RNAs were identified in a search for unusually hydrophobic RNAs. Similar approaches based on

The few modifications mentioned here are not meant as a conclusive list but rather to highlight a few cases where the connections may be more obvious yet widely understudied. In the end the next challenge is to integrate the fields of modomics and metabolomics to establish where the lines are drawn. It is therefore the use of multiple approaches that will lead to the elucidation of the connectivity between modifications and other cellular metabolic programs that will place RNA modifications at the heart of key regulatory loops controlling cellular systems.

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